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DNA VACCINES: Immunology, Application, and Optimization*

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■ **Abstract** The development and widespread use of vaccines against infectious agents have been a great triumph of medical science. One reason for the success of currently available vaccines is that they are capable of inducing long-lived antibody responses, which are the principal agents of immune protection against most viruses and bacteria. Despite these successes, vaccination against intracellular organisms that require cell-mediated immunity, such as the agents of tuberculosis, malaria, leishmaniasis, and human immunodeficiency virus infection, are either not available or not uniformly effective. Owing to the substantial morbidity and mortality associated with these diseases worldwide, an understanding of the mechanisms involved in generating long-lived cellular immune responses has tremendous practical importance. For these reasons, a new form of vaccination, using DNA that contains the gene for the antigen of interest, is under intensive investigation, because it can engender both humoral and cellular immune responses. This review focuses on the mechanisms by which DNA vaccines elicit immune responses. In addition, a list of potential applications in a variety of preclinical models is provided.

INTRODUCTION

The concept of vaccination was demonstrated over 200 years ago when Jenner showed that prior exposure to cowpox could prevent infection by smallpox. Over the last century, the development and widespread use of vaccines against a variety of infectious agents have been a great triumph of medical science. Despite these successes, vaccines for many pathogens throughout the world, including human immunodeficiency virus (HIV) and the agents of malaria and tuberculosis, are

either ineffective or unavailable. One of the impediments to successful vaccination against the aforementioned infectious agents is that they likely require a cellular immune response for protection. In this regard, although all currently licensed vaccines are efficient at inducing antibody responses, only vaccines derived from live attenuated organisms induce cellular immunity efficiently. It should be noted, however, that widespread use of live attenuated vaccines might be precluded by practical constraints such as manufacturing and safety concerns. Thus, the demonstration over the last decade that plasmid DNA vaccines can induce both humoral and cellular immune responses in a variety of murine and primate disease models has engendered considerable excitement in the vaccine community.

The historical basis for DNA vaccines rests on the observation that direct in vitro and in vivo gene transfer of recombinant DNA by a variety of techniques resulted in expression of protein. These approaches included retroviral gene transfer, using formulations of DNA with liposomes or proteoliposomes (1–3), calcium phosphate-coprecipitated DNA (4), and polylysine-glycoprotein carrier complex (5). In the seminal study by Wolff et al of "plasmid or naked" DNA vaccination in vivo, it was shown that direct intramuscular inoculation of plasmid DNA encoding several different reporter genes could induce protein expression within the muscle cells (6). This study provided a strong basis for the notion that purified/recombinant nucleic acids ("naked DNA") can be delivered in vivo and can direct protein expression. These observations were further extended in a study by Tang et al (7), who demonstrated that mice injected with plasmid DNA encoding hGH could elicit antigen-specific antibody responses. Subsequently, demonstrations by Ulmer et al (8) and Robinson et al (9) that DNA vaccines could protect mice or chickens, respectively, from influenza infection provided a remarkable example of how DNA vaccination could mediate protective immunity. The mouse study further documented that both antibody and $CD8^+$ cytotoxic T-lymphocyte (CTL) responses were elicited (8), consistent with DNA vaccines stimulating both humoral and cellular immunity.

DNA vaccination might provide several important advantages over current vaccines (Table 1). (*a*) DNA vaccines mimic the effects of live attenuated vaccines in their ability to induce major histocompatibility complex (MHC) class I-restricted $CD8^+$ T-cell responses, which may be advantageous compared with conventional protein-based vaccines, while mitigating some of the safety concerns associated with live vaccines. (*b*) DNA vaccines can be manufactured in a relatively cost-effective manner and stored with relative ease, eliminating the need for a "cold chain" (the series of refrigerators required to maintain the stability of a vaccine during its distribution). In light of these potential advantages, this review focuses on the mechanisms by which DNA vaccines induce immune responses. In addition, we have provided a table of diseases for which DNA vaccines are effective in animal models. For additional information on DNA vaccination with an emphasis on viral infections, we refer to the recent review

TABLE 1 Comparative analysis of various vaccine formulations

		DNA vaccine	Live attenuated	Killed/protein subunit
Immune response				
Humoral	B cells	+ + +	+ + +	+ + +
Cellular	$CD4^+$	+ + + Th1[a]	+/− Th1	+/− Th1
	$CD8^+$	+ +	+ + +	−
Antigen presentation		MHC class I & II	MHC class I & II	MHC class II
Memory	Humoral	+ + +	+ + +	+ + +
	Cellular	+ +	+ + +	+/−
Manufacturing				
	Ease of development and production	+ + + +	+	+ +
	Cost	+ + +	+	+
	Transport/Storage	+ + +	+	+ + +
Safety		+ + +[b]	+ +[c]	+ + + +

[a]Th2 responses can be induced by gene gun immunization in mice.
[b]Data available only from Phase 1 trials.
[c]Live/attenuated vaccines may be precluded for use in immunocompromised patients and certain infections such as HIV.

by Robinson & Pertmer (10). Finally, a comprehensive web site on DNA established by Whalen (10a) can be found at www.genweb.com/dnavax.html.

REQUIREMENTS FOR A DNA VACCINE VECTOR

There are several factors that influence the type of immune response induced by DNA vaccination. This section outlines the two basic elements of a DNA vaccine that influence the transcription and modulation of the immune responses. A more comprehensive discussion of how the plasmid DNA can be optimized for a specific type of immune response is presented in a later section.

Expression Plasmid Backbone

DNA vaccines consist of the foreign gene of interest cloned into a bacterial plasmid (Figure 1). The plasmid DNA is engineered for optimal expression in eukaroytic cells. Requisites include (*a*) an origin of replication allowing for growth in bacteria (the *E. coli*:ColEI origin of replication in PUC plasmids is most commonly used for this purpose, because it provides large copy numbers in bacteria with high yields on purification); (*b*) a bacterial antibiotic resistance gene (this allows for plasmid selection during bacterial culture; the ampicillin resistance gene, the most common resistance gene used for studies in mice, is precluded for

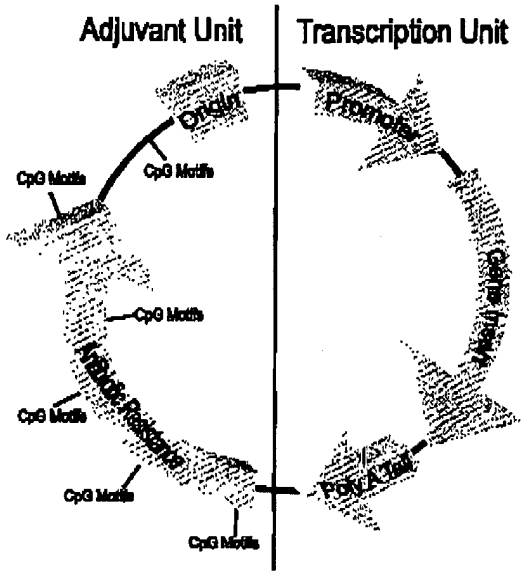

Figure 1 Schematic for the basic requirements of a plasmid DNA vector. The essential features for a plasmid DNA vector include a transcriptional unit, which consists of a viral promoter (i.e. cytomegalovirus), an insert containing the antigen, and transcription/termination sequences (Poly A). The other essential components include a bacterial origin of replication and antibiotic resistance gene, allowing for growth and selection in bacteria. The adjuvant properties of a plasmid vector are highly influenced by the number of CpG motifs within the plasmid backbone.

use in humans, and kanamycin is often used); (*c*) a strong promoter for optimal expression in mammalian cells (for this, virally derived promoters such as from cytomegalovirus (CMV) or simian virus 40 provide the greatest gene expression); and (*d*) stabilization of mRNA transcripts, achieved by incorporation of polyadenylation sequences such as bovine growth hormone (BGH) or simian virus 40.

Contribution of Immunostimulatory Cytidine-Phosphate-Guanosine Motifs

In addition to the requirements outlined above, DNA vaccines also contain specific nucleotide sequences that play an important role in the immunogenicity of these vaccines. Yamamoto et al were the first to report that synthetic oligodeoxynucleotides (ODNs) with sequences patterned after those found in bacterial DNA could activate natural killer cells to secrete interferon (IFN)-γ (11). They hypothesized that palindromic sequences present in the synthetic ODNs were

responsible for this stimulation. More recently, it was shown that a specific sequence motif present in bacterial DNA elicited innate immune responses characterized by the production of interleukin (IL)-6, IL-12, tumor necrosis factor (TNF)-α, IFN-γ, and IFN-α (12–14). This motif consists of an unmethylated cytidine-phosphate-guanosine (CpG) dinucleotide with appropriate flanking regions. In mice, the optimal flanking region is composed of two 5' purines and two 3' pyrimidines (14, 15). Such motifs are 20-fold more common in microbial than mammalian DNA, owing to differences in frequency of use and the methylation pattern of CpG dinucleotides in prokaryotes vs eukaryotes (16, 17). CpG motifs directly activate B cells to proliferate or secrete antibody (15). In addition, they directly induce professional antigen-presenting cells [APCs; i.e. macrophages and dendritic cells (DCs)] to secrete cytokines (12, 18, 19). Natural killer (NK) cells are indirectly activated by CpG motifs through cytokines induced by APCs (20). Finally, T cells are also stimulated directly or indirectly by CpG motifs, depending on their baseline activation state (21). Because CpG motifs have such a prominent role in enhancing the immune response after DNA vaccination, a more detailed summary of their role is highlighted below in the section discussing approaches to vaccine optimization.

IMMUNOLOGY OF DNA VACCINATION

An important first step in the rational design of a vaccine is to understand the immune correlates of protection. For most viral and bacterial infections, primary protection is mediated by a humoral immune response (production of antibodies). For intracellular infections such as *Mycobacterium tuberculosis, Leishmania major,* and other parasites, protection is mediated by cellular immunity. Moreover, for some diseases [e.g. human immunodeficiency virus (HIV) infection, herpes, and malaria], both humoral and cellular responses are likely to be required. The cellular immune response comprises primarily $CD4^+$ and $CD8^+$ T cells. These cells recognize foreign antigens that have been processed and presented by APCs in the context of MHC class II or class I molecules, respectively. Exogenous antigens provided by killed/inactivated pathogens, recombinant protein, or protein derived from live vaccines are taken up by APCs by phagocytosis or endocytosis and presented by MHC class II molecules to stimulate $CD4^+$ T cells, which can help generate effective antibody responses. In contrast, MHC class I molecules associate with antigens synthesized within the cytoplasm of the cell (with rare exceptions) and are generally elicited by live or DNA vaccines. From an immunologic standpoint, based on the broad range of effector cells generated and the memory responses they induce, live attenuated vaccines represent the vaccines of choice for those diseases requiring both humoral and cellular responses (Table 1). From a practical and safety standpoint, however, live or live attenuated vaccines raise several issues that can preclude their widespread use. In this regard, DNA vaccines—which resemble live attenuated vaccines in their ability to induce

both humoral and cellular responses—may prove to be useful alternatives. In the next section, the mechanism by which DNA vaccines induce specific types of immunity is discussed.

Mechanism of Antigen Presentation

One intriguing aspect of DNA vaccination involves the mechanism by which the antigen encoded by the foreign gene introduced into the bacterial plasmid is processed and presented to the immune system. Studies demonstrate that the quantity of antigen produced in vivo after DNA inoculation is in the picogram to nanogram range. Given the relatively small amounts of protein synthesized by DNA vaccination, the most likely explanation for the efficient induction of a broad-based and sustained immune response is the immune-enhancing properties of the DNA itself (i.e. CpG motifs) and/or the type of APC transfected. There are at least three mechanisms by which the antigen encoded by plasmid DNA is processed and presented to elicit an immune response: (*a*) direct priming by somatic cells (myocytes, keratinocytes, or any MHC class II-negative cells); (*b*) direct transfection of professional APCs (i.e. DCs); and (*c*) cross-priming in which plasmid DNA transfects a somatic cell and/or professional APC and the secreted protein is taken up by other professional APCs and presented to T cells. These three mechanisms are highlighted in Figure 2.

Direct Transfection of Professional Antigen-Presenting Cells—Bone Marrow-Derived Cells Directly Mediate Cellular Immune Responses after DNA Vaccination Several elegant studies with bone marrow-chimeric mice have conclusively demonstrated that bone marrow-derived APCs play a key role in the induction of the immune response after DNA vaccination. In these studies, parent into F1 bone marrow-reconstituted mice created a mismatch between the haplotypes of somatic cells and bone marrow-derived cells. The immune response generated on subsequent DNA immunization was found to be restricted to the haplotype of reconstituted bone marrow, providing conclusive evidence that bone marrow-derived cells were responsible for priming immune responses after DNA vaccination (22–24).

Dendritic Cells Are the Principal Cells Initiating the Immune Response after DNA Vaccination The above findings were further extended to evaluate the cellular mechanisms responsible for the activation of T cells after DNA immunization. In particular, studies were aimed at defining the specific type of APCs regulating the immune response after DNA vaccination. The first study to address this question showed that isolated DCs but not B cells or keratinocytes from DNA-vaccinated mice were able to efficiently present antigen to T cells in vitro (25). Moreover, in the same study it was estimated that only a small proportion of the DCs (0.4%) was transfected with plasmid DNA (25). Similar results were obtained in two additional studies in which the injection of DNA led to direct

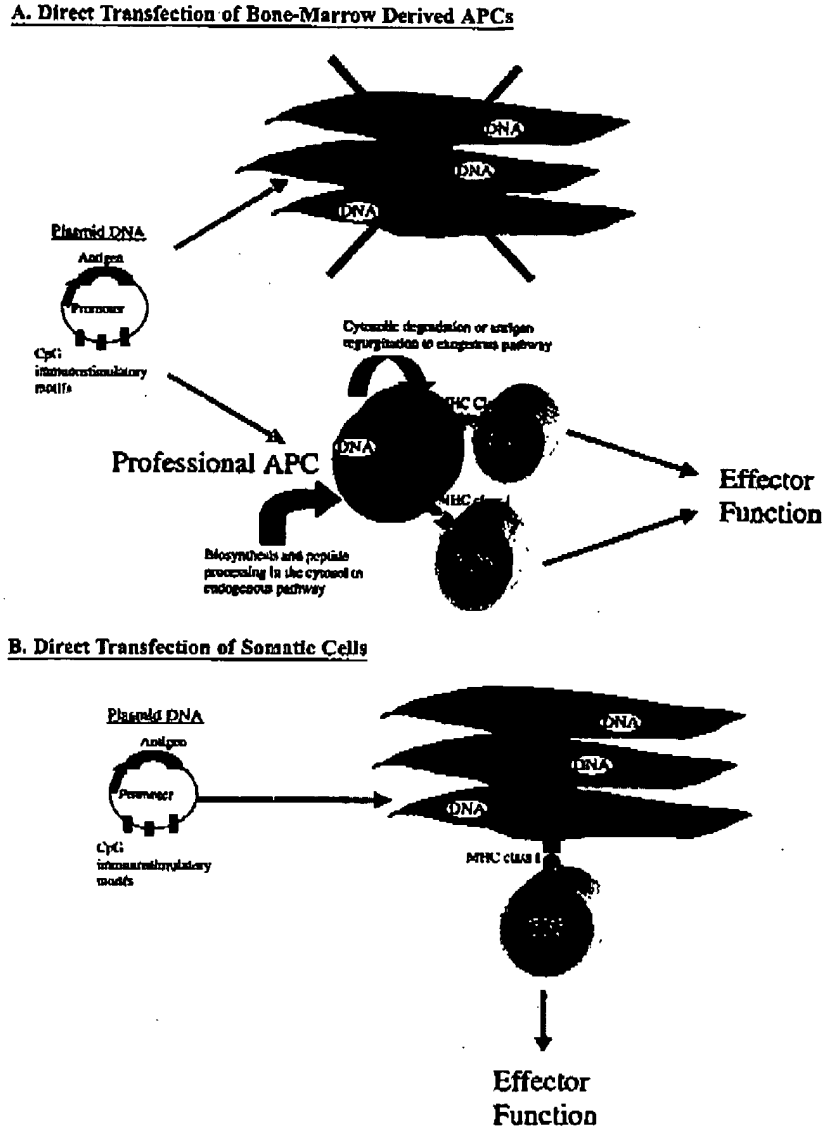

Figure 2 Legend under Figure 2c.

C. Cross Priming

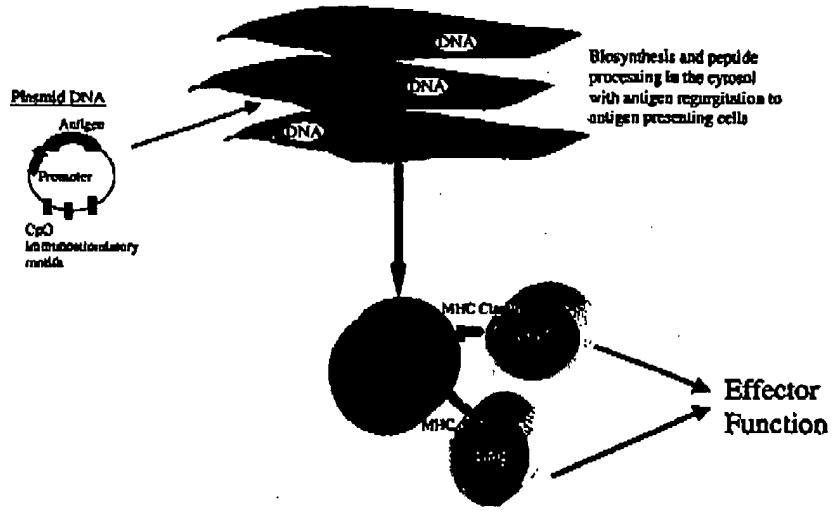

Figure 2 Mechanisms of antigen presentation after DNA immunization. A. Bone marrow-derived antigen-presenting cells (APCs)s mediate immune responses after DNA vaccination. Injection of plasmid DNA leads to direct transfection of a small number of DCs that present antigen to T cells. B. Direct priming of immune responses by somatic cells (myocytes, keratinocytes, or any major histocompatibility complex class-II–negative cells). This result could occur after injection of plasmid DNA into muscle or skin, leading to protein production and presentation to T cells by the somatic cells themselves. Alternatively, C. Protein production by transfected somatic cells may be taken up by professional APCs, leading to T-cell activation (cross-priming).

transfection of small numbers of DCs (26, 27). It is notable that in both of these studies there was general activation and migration of large numbers of DCs that were not transfected. Finally, direct in vivo visualization of antigen-expressing DCs from draining lymph nodes after gene gun vaccination was demonstrated in a separate study in which gold particles and protein expression from a reporter gene could be co-localized within a cell that had morphologic indices consistent with a DC (28). Taken together, the preponderance of data clearly demonstrates that DCs play a key role in induction of the immune response after DNA vaccination. Furthermore, these data suggest that the predominant contribution to priming immune responses after DNA vaccination involves a small number of directly transfected DCs. Additionally, as noted above, the question arises whether the enhancement in the number of migrating DCs not directly transfected with DNA, seen in many studies, could also present antigen via additional mechanisms such as cross-priming (see below).

Direct Priming of Somatic Cells—Skin vs Muscle The initial seminal study by Wolff et al (6) demonstrating the success of "plasmid or naked" DNA vaccination in vivo involved the direct intramuscular inoculation of plasmid DNA, leading to expression of protein within the transfected cell. The other important study by Ulmer et al (8), showing that direct intramuscular inoculation of plasmid DNA induced a strong $CD8^+$ CTL to influenza nucleoprotein, provided the first evidence that cellular responses could be induced in vivo and have a potentially important protective role. These and several additional studies suggested that muscle cells were critically involved in the initiation of immune responses after DNA vaccination. One conceptual difficulty with this premise is that, although muscle cells express MHC class I molecules, they do not express other cell surface molecules (i.e. CD80 and CD86) that are critical in optimizing T-cell priming. Therefore, they are not likely to be as efficient at presenting antigen as are DCs. This difficulty raised a question about the exact role that muscle cells play in the induction of cellular immune responses after intramuscular DNA vaccination. To address whether expression of antigen by myocytes was sufficient to induce protective immunity in vivo, it was shown that transfer of stably transfected myoblasts expressing an influenza nucleoprotein protected mice from infectious challenge (29). Although these data suggested that expression of viral protein by muscle cells in vivo is sufficient for CTL-mediated protection, the question of whether CTLs were induced directly by myocytes expressing protein directly or by transfer of protein from myocytes to professional APCs (cross-priming) remained open.

Experiments were undertaken to directly test whether muscle cells alone are sufficient to prime immune responses. In one study, using bone marrow chimeras to examine the contribution of bone marrow- and non-bone marrow–derived cells to CTL priming, it was shown that antigen-specific CTL responses could be induced by non-bone marrow–derived (muscle) cells only when mice were vaccinated with DNA encoding the antigen and CD86 (30). By contrast, in a separate study with a plasmid DNA encoding a different antigen, it was shown that plasmids encoding CD86, IL-12, or granulocyte/macrophage colony-stimulating factor DNA failed to induce muscle cells to prime for CTL responses (31). Taken together, although these studies both show that muscle cells alone are not efficient at priming immune responses, one study does suggest that muscle cells expressing CD86 are sufficient to induce a response. Finally, the finding that removing the muscle immediately (within 10 min) after immunization does not alter the subsequent immune response (32) provides additional evidence that injected plasmid DNA is likely to gain access to the lymphatic or circulatory system, thus obviating the need for transfection of muscle cells at the site of injection.

For other somatic cells, it has been shown that keratinocytes and Langerhans cells constitute the major cell types transfected by plasmid DNA after injection into the skin (33, 34). In contrast to the data mentioned above regarding removing muscle, immediate removal of skin after DNA vaccination prevented development of immune responses (32). Moreover, in a separate study, it was shown that

transplantation of vaccinated skin <12 h postvaccination could elicit an immune response in naive animals (35). By contrast, little or no immune response could be initiated when the period of transplantation exceeded 24 h. These data suggest that cells that migrated from the epidermis within 24 h of immunization induced the primary immune response after DNA vaccination. Finally, it was shown that the magnitude of the primary immune response increased when the vaccination site was left intact (35). Taken together, these data suggest that antigen-expressing nonmigratory cells such as keratinocytes may continue to produce antigen to augment the immune response (27, 35).

Cross-Priming As discussed above, secreted or exogenous proteins undergo endocytosis or phagocytosis to enter the MHC class II pathway of antigen processing to stimulate CD4+ T cells. Endogenously produced proteins/peptides (e.g. viral antigens) are presented to the immune system through an MHC class I-dependent pathway to stimulate naive CD8+ T cells. Although peptides derived from exogenous sources are generally excluded from presentation on MHC class I molecules, there are now several examples showing that this can occur in vivo (36–40). Moreover, the concept of cross-priming, in which triggering of CD8+ T-cell responses can occur without de novo antigen synthesis within the APCs, provides an additional mechanism by which DNA immunization can enhance immune responses. During cross-priming, antigen or peptides (both MHC class I and II) generated by somatic cells (myocytes or keratinocytes) can be taken up by professional APCs to prime T-cell responses. The demonstration that transfer of myoblasts expressing an influenza nucleoprotein into F1 hybrid mice induced CTL responses restricted by the MHC haplotype of the recipient mice provided the first evidence that transfer of antigen from myocytes to professional APCs can occur in vivo in the absence of direct transfection of bone marrow–derived cells (29, 41). In addition, cross-priming can occur when professional APCs process secreted peptides or proteins from somatic cells and/or other APCs by phagocytosis of either apoptotic or necrotic bodies (42, 43). This is supported by a study showing that cross-priming of DCs occurred when keratinocytes expressing antigen were exposed to irradiation in vitro, leading to cell death (27).

In summary, the overwhelming evidence suggests that bone marrow-derived APCs, but not somatic cells, directly induce immune responses after DNA vaccination; however, because somatic cells such as myocytes or keratinocytes constitute the predominant cells transfected after DNA inoculation via muscle or skin injection, respectively, these cells may serve as a reservoir for antigen. Thus, somatic cells can be important in the induction of immune responses via cross-priming and may play a role in augmenting and/or maintaining the response.

Cellular Immunity

CD4+ T Helper Cell Responses CD4+ T cells play a central role in immune homeostasis. There are at least three major functions that CD4+ T cells can mediate. First, activated CD4+ T cells have a critical role in promoting B-cell survival

and antibody production through CD40L-CD40 interactions (44). Second, CD4+ T cells, through production of IL-2 and/or through CD40L-CD40 costimulation, provide helper function to CD8+ T cells (45–47). Finally, CD4+ T cells secrete a myriad of cytokines that have profound immunoregulatory effects in many disease states. In this regard, it has been demonstrated that activated CD4+ T cells can be segregated into two distinct subsets based on their production of certain cytokines (48). For example, T-helper-1 (Th1) cells exclusively produce IFN-γ, whereas CD4+ T cells that exclusively produce IL-4, IL-5, and IL-13 are designated as T-helper-2 (Th2) cells. Although several factors have been shown to influence the differentiation of Th1- and Th2-type cells, the cytokine milieu present at the time of initial T-cell priming appears to be the most important (48, 49). Thus, the presence of IL-12 facilitates differentiation toward a Th1 phenotype, whereas the presence of IL-4 allows for Th2 differentiation.

Because CpG motifs present in bacterial DNA can trigger the immune system to induce a variety of proinflammatory cytokines including IL-12, it would follow that the generation of Th1 responses may be a general property of DNA vaccines. Indeed, DNA vaccination has been successfully applied to several animal models of infection in which induction of a Th1 response correlates with protection (e.g. tuberculosis and leishmaniasis). DNA vaccination has also proven to be successful in a mouse model of respiratory syncytial virus infection, in which it is likely that antibodies correlate with protection. It is important that, in this infection, killed/inactivated vaccines induced a Th2-type response, which was associated with unfavorable pathology and outcome (50). This is a striking example in which DNA vaccination (by preferentially inducing a Th1 response) has a definite advantage over a formalin-inactivated respiratory syncytial virus vaccine by changing the qualitative immune response (51). Additional evidence that DNA vaccination favors a Th1 response stems from the observation that the predominant immunoglobulin (Ig) isotype detected after DNA vaccination is IgG2a (52). Of note, however, is that, under certain circumstances, DNA vaccines can also induce Th2 responses. Perhaps the best example of this involves using the gene gun method of immunization. Pertmer et al (53) first demonstrated increased IL-4 production in mice repetitively immunized by gene gun, while production of IFN-γ concomitantly decreased. As a further correlate, it was shown that the predominant Ig isotype generated after repetitive gene gun immunization was IgG1, whereas the predominant Ig isotype generated after intramuscular immunization was IgG2a (53). These observations were extended by the work of Feltquate et al (54), who substantiated the finding that different predominant T helper-type cytokines were generated by gene gun versus intramuscular DNA immunization. Preferential Th2 responses occurred whether DNA plasmids on gold beads "shot" into the skin or into surgically exposed muscle. Taken together, these data suggest that the use of the gene gun has a powerful influence on the induction of Th2 response regardless of the route of immunization. A potential explanation for why Th2-type responses are induced by gene gun is that the gun delivers plasmid DNA directly into cells, thus bypassing surface interaction of

CpG motifs, present in the plasmid backbone, with the APCs to mediate the release of proinflammatory Th1-type cytokines. Moreover, because DNA vaccines target DCs, it is possible that different methods of immunization could target different subsets of DCs that have been shown to preferentially bias helper T cell responses (55–59). Finally, there is some evidence that the nature of the antigen used (secreted vs intracellular) can preferentially bias T-helper responses (60, 61). It should be noted, however, that these studies used antibody subtypes rather than direct measurement of cytokine production as a surrogate for T-helper responses.

The potential of DNA vaccines to strongly influence CD4+ T-helper cell responses has several practical implications. For infectious diseases, the ability of DNA vaccines to preferentially generate Th1 responses may be particularly useful for preventing intracellular infections requiring Th1 immunity or for modulating ongoing immune responses to optimize intracellular killing. First, in terms of influencing an ongoing response, it was shown that CpG ODN treatment could strikingly enhance IFN-γ and diminish IL-4 production in BALB/c mice that were already infected with *Leishmania major,* suggesting that Th1-type responses could be induced in the course of inducing ongoing Th2 response (62). Additionally, a Th1 response generated by DNA immunization may prevent or limit an ongoing Th2 response, for example, in allergic or asthmatic diseases. This was demonstrated in a study by Raz et al (63), who showed that a Th2 response (reflected by antigen-specific production of IL-4 and IgE antibody) generated by vaccination with β-galactosidase (β-gal) protein plus alum immunization could be altered (decreased IL-5 and IgE production) when these mice were boosted with plasmid DNA encoding β-gal. These data raise the broader question of whether immunotherapy with DNA vaccines affects already differentiated Th2 cells at a single-cell level or influences naive and/or activated but uncommitted CD4+ T cells toward Th1 cytokine production at the population level. In addition to its ability to influence an established Th2 response, in a rat model of allergic hyperresponsiveness, it was demonstrated that injection of plasmid DNA encoding a house dust mite allergen prevented the induction of IgE and reduced airway hyperreactivity (64). In that study, the suppression of allergic responses could be transferred by CD4-depleted T cells. These findings raise the possibility that CD8+ T cells can suppress IgE production and confirm the ability of DNA vaccines to induce both MHC class I- and class II-restricted responses (64). Finally, in experimental models of autoimmune disease, it appears that type I cytokine production (IL-12 or IFN-γ) correlates with disease progression. Therefore, it might be expected that DNA vaccination would not be useful for preventing or limiting ongoing autoimmune diseases associated with Th1 responses (65), yet it was demonstrated that vaccination of mice with DNA encoding a gene for a pathogenic T-cell receptor (Vβ8.2) for experimental autoimmune encephalitis (EAE) actually protected mice. Protection was associated with a reduction in the Th1 response and increase in the Th2 response (66). The mechanism for this novel observation remains to be elucidated.

Cytotoxic T-Lymphocyte Responses As noted above, one of the major advantages of DNA vaccines is the ability to generate antigens endogenously, making them accessible to $CD8^+$ T cells via an MHC class I pathway (8). Although CD8 responses are also generated by live vaccines, they are difficult to induce with conventional protein-based vaccines. Moreover, owing to the potential safety concerns about certain live viral vaccinations, the induction of $CD8^+$ CTL responses after DNA vaccination may represent a principal advantage of this type of vaccine approach. In addition, because plasmid DNA encoding an antigen can be easily modified, this method of vaccination allows for optimization of both the qualitative and quantitative aspects of CTL responses (see section on vaccine optimization below).

While it is clear that DNA vaccination is an effective method of inducing $CD8^+$ T-cell responses, there are at least two critical issues concerning the ability of this vaccine approach to mimic the responses of those achieved with live viral vaccines. The first point relates to the magnitude of the CTL response, whereas the second relates to the generation of CTL responses against dominant and subdominant epitopes. In three separate studies of DNA vaccination against lymphocytic choriomeningitis virus (LCMV) infection, mice inoculated with DNA encoding an LCMV protein generated no detectable CTL responses before infectious challenge (67–69). DNA-vaccinated mice, however, were protected from challenge with LCMV. Furthermore, in one of these studies, it was shown that mice inoculated with live LCMV had CTL activity that was immediately detectable ex vivo (69). Taken together, these data show that, in the LCMV mouse model, although live viral infection but not DNA vaccination induced a detectable frequency of effector CTLs immediately ex vivo, DNA vaccination did induce low numbers of precursor CTLs that expanded in vivo after infectious challenge sufficient for protection. In contrast to the LCMV model, the frequency of CTL precursors from cells of mice that were vaccinated with plasmid DNA encoding a Sendai virus nucleoprotein were comparable to those elicited by live Sendai virus infection in a previous report (70). Similarly, in a separate study, it was shown that CTLs generated from mice vaccinated with plasmid DNA encoding influenza nucleoprotein were comparable to those derived from mice that were infected with influenza virus (71). Thus, depending on the antigen and viral model system used, DNA vaccination can elicit CTL responses that are similar to live viral infection after short-term in vitro culture. Perhaps the critical issue of whether DNA vaccination is similar to live viral infection will be resolved by comparing the effector CTL responses immediately ex vivo without any further in vitro culturing. Current techniques, using MHC class I-specific tetramers and intracellular cytokine staining, should clarify this question. These issues are relevant to the optimization of vaccines for infections such as HIV infection or malaria, in which a high precursor frequency of effector CTLs at the time of infection may be required to limit dissemination of infection.

Although the magnitude of CTLs induced by DNA vaccination may be sufficient for protection after infectious challenge, an additional consideration is

whether DNA vaccines can elicit the same breadth of response as that induced by natural infection. In this regard, although the number of epitopes available in a primary CTL response is relatively large after viral infection, the effector CTL responses selected by the host are often limited to a few dominant epitopes. Additionally, responses to subdominant epitopes may be important in mediating an effector role in the absence of CTL responses to a dominant epitope. For instance, two separate studies showed that DNA vaccines encoding an influenza or Sendai virus nucleoprotein were able to elicit CTL responses against both dominant and subdominant epitopes (70, 71). These data suggest that DNA vaccines can elicit broad memory responses to multiple epitopes. In this aspect, DNA vaccines resemble live viral vaccines by inducing a broad precursor CTL frequency and memory.

Humoral Responses

Immunization with plasmid DNA can induce a strong antibody response to a variety of proteins in animal species, including mice, non-human primates, and, most recently, human subjects. Moreover, the humoral response generated by DNA vaccination has been shown to be protective in several animal models in vivo. Because conventional protein vaccines also induce protective antibody responses, it is useful to not only review the mechanism by which DNA vaccines induce antibody responses but also highlight potential differences and determine whether DNA vaccination offers any advantages compared with other types of vaccination.

Dose Response and Kinetics of Antibody Response Induced by DNA Vaccination With regard to the quantitative aspect of antibody production after DNA vaccination, it was shown in two separate studies with DNA-encoding influenza hemagglutinin antigen that the antibody responses peaked and reached a plateau between 4 and 12 weeks after a single DNA immunization in mice (72, 73). Furthermore, antibody production is increased in a dose-responsive manner with either a single injection or multiple injections of DNA by various routes of immunization (72, 73). Although dosage and frequency of immunizations may affect the kinetics and magnitude of the response, it is interesting that single or multiple injections with an optimal dose of DNA did not significantly affect the amount of antibody produced once a plateau had been reached (72, 73). Finally, although the duration of the antibody response can be long lived [significant serum levels were present up to 1.5 years postvaccination (34, 72)], this duration is highly variable and depends on the model system and vaccine used.

Comparison of Antibody Responses between DNA Vaccination and Protein or Live Infection: Effects on Avidity, Magnitude, Isotype, and Induction of Neutralizing Antibody As noted above, peak antibody responses after DNA vaccination occur 4–12 weeks postvaccination. Most studies comparing antibody production after DNA, protein, and live virus immunization use this time range.

In comparing humoral responses in mice vaccinated with DNA H1 hemagglutinin and mice immunized with a sublethal viral challenge with H1N1 influenza, the amount of antibody produced was substantially greater and peaked more rapidly in the sublethally infected mice than in the DNA-vaccinated mice (74). Similarly, in a separate study comparing the antibody response to DNA encoding the hemagglutinin (HA) antigen and live influenza infection, the antibody titers in mice vaccinated with live influenza were higher than in DNA-vaccinated mice, although this result was seen with only certain antibody isotypes (72). In comparing the antibody response elicited by vaccination with DNA and protein, it was shown that antibody titers and avidity were significantly lower in mice vaccinated with DNA encoding a malarial surface protein than in those vaccinated with the protein alone (75). By contrast, in one study directly comparing the kinetics of antibody response after vaccination with DNA-encoding ovalbumin (OVA) and OVA protein, there did not appear to be a difference in total OVA-specific antibody production when DNA was administered intradermally at 2 or 4 weeks postvaccination (76). In this study, antibody induced by DNA had a higher avidity than that induced by protein.

The antibody subtypes induced by DNA vaccination include IgG, IgM, and IgA. Moreover, as noted in the previous section, DNA vaccination generally enhances Th1 cytokine production. Because cytokines such as IL-4 and IFN-γ can direct IgG1, and IgG2a production, respectively, it follows that the subclass of antibodies generated by pDNA vaccination will be biased toward IgG2a production. While this appears to be a general property of DNA vaccination in mice, it has been shown that DNA encoding secreted antigen generated higher levels of IgG1 than did membrane-bound antigen (60). Moreover, as noted above, the route of DNA vaccination (gene gun) can also preferentially bias toward IgG1 production (54).

Finally, the ability of DNA vaccines to generate neutralizing antibodies suggests that antigen expressed in vivo after DNA vaccination can assume a native configuration. In this regard, the ability of plasmid DNA encoding influenza HA to generate neutralizing antibody suggests that HA was present in its native form, because the epitopes of HA that are recognized by these antibodies are formed by noncontiguous regions within HA. Thus, DNA vaccines may generate antibody responses that more closely resemble those seen after natural infection and provide a potential advantage over conventional protein vaccines. Since some recombinant proteins may lack linear determinants or conformational epitopes required for efficient generation of neutralizing antibodies. Data to support this were shown for mice immunized with a DNA vaccine encoding HIV gp120, in which sera contained antibodies reactive to linear epitopes within the V3 region of gp120 whereas sera from mice immunized with recombinant gp120 contained much lower levels of V3-specific antibodies (77). Similar results were observed with a rabbit model of papilloma virus (CRPV) infection. In this model, immunization with plasmid DNA encoding a major capsid protein L1 induced neutralizing antibody. In that study, adsorption experiments with native L1 or

denatured LI protein suggested that vaccination with plasmid DNA encoding LI elicited conformationally specific neutralizing antibody (78).

Memory Immunity

The hallmark of any successful vaccine is the ability to induce long-term memory. Current vaccines—whether live attenuated or protein subunit—are successful at generating durable humoral responses. For diseases requiring cellular immunity such as parasitic, mycobacterial, and certain viral infections, however, it is not yet clear how memory responses are generated and maintained after vaccination. Regarding humoral immunity, it has been shown that mice vaccinated with DNA encoding an HA antigen had levels of anti-HA antibodies comparable with or greater than those from convalescent sera of previously infected mice that persisted over 1 year (34, 72). In other studies, however, plasmid DNA encoding a nucleoprotein of the LCMV virus administered by gene gun (69) or intramuscularly (67) either failed to give appreciable antibody responses before challenge or the responses had waned by 4 months postimmunization. Taken together, these results indicate that, while DNA vaccination can be effective at inducing long-term antibody responses, this effect may depend on the type of antigen used in the vaccine.

In terms of cellular immunity, it was recently shown that the frequency of antigen-specific $CD4^+$ T cells as measured by proliferation remained elevated for ≤40 weeks postvaccination. Of interest, antigen was detectable only for 2 weeks postvaccination in DCs in the draining lymph nodes but for ≤12 weeks in keratinocytes (27). Moreover, a functional assay performed in vivo appeared to demonstrate no source of antigen present in the spleen or lymph nodes 20 days postvaccination. Taken together, these data showed that antigen-specific $CD4^+$ T cells are activated in the draining lymph nodes and migrate to the spleen, where they can persist for up to 40 weeks in the absence of detectable antigen (27). More definitive evidence showing that DNA can induce long-lived Th1 effector responses in vivo involved a mouse model of *L. major* infection. This study demonstrated that vaccination with plasmid DNA encoding a specific leishmanial antigen is more effective than vaccination with leishmanial protein plus IL-12 protein in maintaining antigen-specific Th1 cells capable of controlling *L. major* infection (79). These data provided evidence that DNA vaccination can induce long-term Th1 responses and suggested that DNA vaccination may be more effective than vaccination with protein plus adjuvant (i.e. IL-12). Reasons for the enhanced efficacy of DNA vaccination over protein and adjuvant may include low levels of persistent antigen and/or IL-12 induced by the CpG in the plasmid DNA.

Induction of Long-Term Cytotoxic-T-Lymphocyte Responses after DNA Vaccination Although few studies have assessed the induction of $CD8^+$ T-cell responses for prolonged periods after DNA vaccination, there is a report showing

that CTL responses could be observed ≤68 weeks after intradermal injection of DNA encoding a nucleoprotein from influenza virus (34). In a separate study, DNA-primed CTL responses to hepatitis B virus envelope proteins could be detected for ≤4 months post-DNA injection (80). It should be noted that these responses were detected only after cells were re-cultured in vitro for several days and then tested. Thus, the relative effectiveness of DNA vaccination for generating fresh, memory effector CTL responses remains to be determined. One other potentially important finding relating to DNA vaccination and induction of memory $CD8^+$ T-cell responses is that CpG motifs are potent stimulators of type-1 interferons. It was originally reported by Tough et al that IFN-α enhances the proliferation of $CD8^+$ T cells expressing a surface marker, consistent with a memory phenotype (81). More recent work showed that CpG DNA appeared to stimulate T cells by inducing type-1 interferons from APCs (82). Although these studies are important in establishing a role for IFN-α in regulating activation of $CD8^+$ T cells, a functional in vivo role for these cells remains to be elucidated.

Mechanisms by Which DNA Vaccinations Induce Sustained Humoral and Cellular Immune Responses There is evidence that long-lived antigen-specific proliferative responses that are induced by DNA vaccination are maintained in the absence of detectable antigen (27). In contrast, one of the original studies on DNA vaccination by Wolff et al showed that intramuscular inoculation of plasmid DNA encoding several different reporter genes resulted in protein expression for >1 year (83). These data raise several possibilities as to how DNA vaccines induce long-term responses: (*a*)antigen is continuously present at low levels sufficient for antigen presentation but below the limit of detection as assessed by polymerase chain reaction or currently available functional assays. Alternatively, plasmid DNA may not be detectable, but synthesized antigen could persist in vivo (i.e. follicular DCs), providing a reservoir to maintain the immune response; (*b*) plasmid DNA as well as antigen are completely gone, and responses are antigen independent (27); and/or (*c*) memory cells generated by DNA vaccines differ qualitatively from those achieved by other forms of vaccination such as protein plus adjuvant.

APPROACHES TO VACCINE OPTIMIZATION

Because different diseases have specific requirements for protective immunity, a rational approach to vaccine optimization would reflect these distinct requirements. Thus, one of the principal advantages of DNA vaccination is the ease with which plasmid DNA can be manipulated to alter the quantitative and qualitative aspects of the immune response. In this section, we discuss the factors that affect the efficiency of DNA vaccines and highlight how DNA vaccines can be influenced or tailored to generate the desired immune response.